ORIGINAL ARTICLES—Laboratory science

Concentration dependent effects of hydrogen peroxide on lens epithelial cells

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Abstract

***Aims*—To evaluate the effects of hydrogen peroxide exposure on the survival and proliferation of cultured lens epithelial cells.**

***Methods*—TOTL-86 cells, a line of rabbit lens epithelial cells, were used. The survival and proliferation of TOTL-86 cells were quantified by a rapid colorimetric assay (MTT assay). To determine the effects of hydrogen peroxide, TOTL-86 cells were exposed to different concentrations of hydrogen peroxide. To determine the effect of cell numbers on the survival and proliferation of TOTL-86 cells at a fixed concentration of hydrogen peroxide, different numbers of cells were plated and exposed to hydrogen peroxide. To determine whether there is a synergistic effect between hydrogen peroxide and EGF, bFGF, PDGF-AA, and insulin, TOTL-86 cells were exposed to hydrogen peroxide combined with one of these growth factors.**

***Results*—High levels (1 mM) of hydrogen peroxide killed TOTL-86 cells and sublethal levels (100 µM) suppressed their proliferation. From 1 nM to 1 µM of hydrogen peroxide, there was a dose dependent increase in the cell numbers. The initial seeded cell number dramatically affected the response to hydrogen peroxide. Although growth factors showed no synergistic effects with hydrogen peroxide on proliferation, both EGF and insulin, but not bFGF or PDGF, rescued TOTL-86 cells from the sublethal effect.**

***Conclusion*—Hydrogen peroxide in cooperation with some growth factors plays an important role in the proliferation of lens epithelial cell.**

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Secondary cataract formation, which is one of the most common and serious complications of cataract surgery,[1 2] represents a wound healing process of the lens epithelial cells remaining after cataract surgery.[3–6] While it is believed that growth factors contribute importantly to the proliferation of residual lens epithelial cells,[7–11] recent studies have shown that hydrogen peroxide also stimulates the growth of a variety of cell types.[12–16] The question then arises as to whether hydrogen peroxide also plays a role in the proliferation of residual lens epithelial cells.

The anterior chamber of the eye is continuously exposed to oxidative stress, and significant levels of hydrogen peroxide have been reported in the aqueous humour of some cataract patients.[17–19] Although cataract researchers have focused on hydrogen peroxide as a possible cause of chronically inflicted damage to lens epithelial cells,[20–24] little is known about hydrogen peroxide as an intracellular signalling molecule in lens epithelial cells.

As a first step, the present in vitro study was undertaken to evaluate the effects of hydrogen peroxide on the growth and survival of lens epithelial cells. We also investigated the synergistic effects of hydrogen peroxide and several growth factors on the growth and survival of lens epithelial cells.

Materials and methods

The experiments were performed on a rabbit lens epithelial cell line, TOTL-86, which we have established and described.[25 26] All cultures were grown in 75 cm^2 culture flasks (Falcon Inc). Eagle's minimum essential medium (E-MEM), fetal calf serum (FCS), and 0.05% trypsin-0.025% EDTA were purchased from Gibco Inc. Dimethyl sulphoxide (DMSO) and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Inc. Epidermal growth factor (EGF),

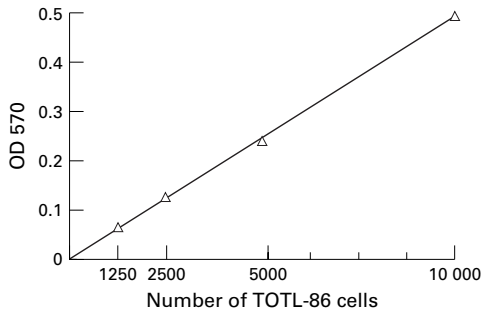

Figure 1 The relation between cell numbers and the optical density (OD). There is a statistically significant linear relation between the number of TOTL-86 cells and the OD at 570 nm (at 1.25 ×10³ cells/well, the OD at 570 nm = 0.065 (SD 0.003): at 2.5 ×10³ cells/well, the OD = 0.126 (0.002): at 5.0 ×10³ cells/well, the OD = 0.239 (0.009): at 10 ×10³ cells/well the OD = 0.492 (0.004). Equation for the line is Y = (4.88 × 10⁻⁵) (OD), p <0.0001.

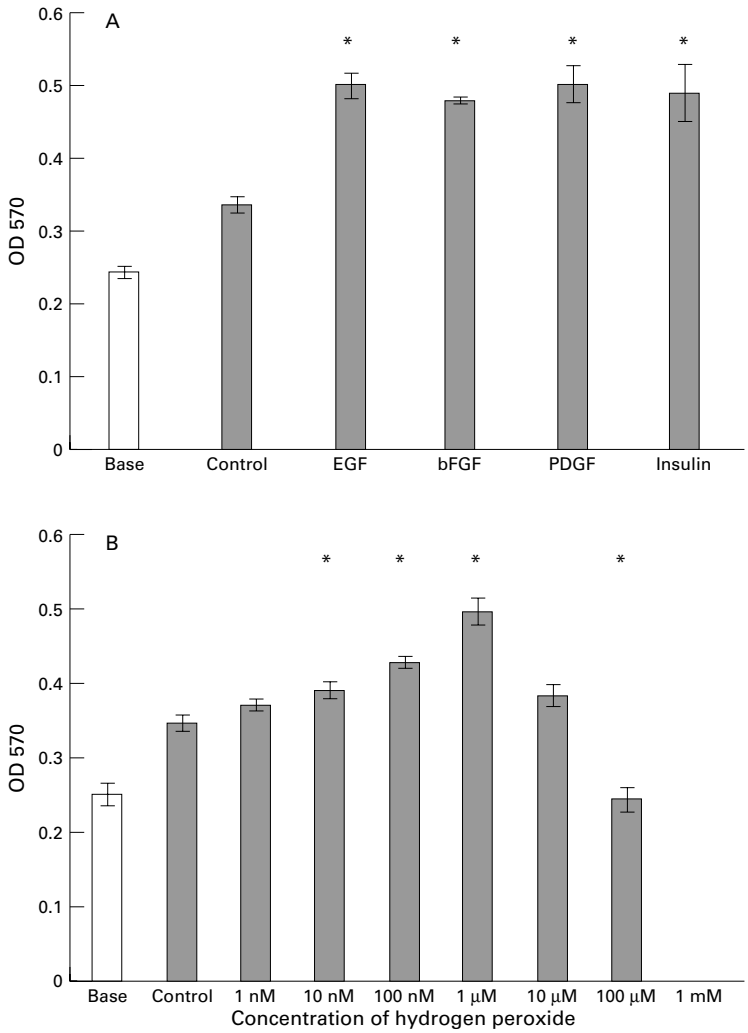

*Figure 2 (A) The effect of four growth factors on the proliferation of TOTL-86 cells. A significant increase in the proliferation of TOTL-86 cells was found with all the growth factors. "Base" is the pretreatment TOTL-86 cells group, and "control" is the TOTL-86 cells added in 1 μl of E-MEM only group. Both base and control have the same meaning in the rest of the figures. Data represent the mean OD (SEM) and * indicates statistical significance compared with control value (control v EGF, p =0.0002: control v bFGF, p <0.0001: control v PDGF, p =0.0004: control v insulin, p =0.0029). (B) The effect of different hydrogen peroxide concentrations on the proliferation of TOTL-86 cells. There is a dose dependent increase in proliferation from 1 nM to 1 μM (control v 1 nM, p=0.0373; control v 10 nM, p = 0.0085; control v 100 nM p = 0.0004; control v 1 μM, p=0.0004). At 10 μM, the growth of TOTL-86 cells was still significantly higher than the controls (control v 10 μM, p = 0.0241) but was less than the optimal concentration. When treated with 100 μM concentration of hydrogen peroxide, the proliferation was significantly suppressed (control v 100 μM, p = 0.0008) but the number of TOTL-86 cells did not differ from the number of cells in the base wells (base v 100 μM, p = 0.5621). A concentration of 1 mM was cytotoxic to TOTL-86 cells.*

platelet derived growth factor AA (PDGF-AA), basis fibroblast growth factor (bFGF), and insulin were also purchased from Gibco Inc.

CELL CULTURE TECHNIQUES

TOTL-86 cells were grown in 75 cm^2 culture flasks in E-MEM with 5% FCS and maintained in a humidified 37°C incubator containing 5% carbon dioxide. The medium was changed every third day until growth was confluent. The cells were then harvested by trypsinisation and replated (passage dilution 1:4).

RAPID COLORIMETRIC ASSAY FOR CELLULAR GROWTH AND SURVIVAL (MTT ASSAY)

The MTT assay is colorimetric assay based on the tetrazolium salt MTT that detects living but not dead cells. The signal generated, the optical density (OD) at 570 nm, is directly proportional to the number of cells.[27 28] MTT was dissolved in phosphate buffered saline (PBS) at 5 mg/ml and filtered to sterilise and remove the small amount of insoluble residue. At a selected time, 10 μl of stock MTT solution was added to all wells for the assay. After a further period of incubation (4 hours), the medium was aspirated from the wells as completely as possible without disturbing the formazan crystals. Then, 100 μl of DMSO was added to each well, and the plates agitated on a plate shaker for 5 minutes. The OD at 570 nm was then read with an ELISA reader (Microplate Reader Model 450; Bio-Rad).

To demonstrate that the OD obtained with reduced MTT is directly related to the number of TOTL-86 cells, we seeded 1250–10 000 cells/well in 100 μl of media into 96 well flat bottomed tissue culture plates (Falcon). The cells were cultured for 16 hours to allow adherence before the MTT assay was performed. In addition, to confirm that OD obtained with reduced MTT certainly represent the living cell numbers in the following experiments, we counted the living cell numbers with a dye exclusion test. In brief, the cells in the other wells were washed, trypsinised, and the number of cells evaluated by direct cell counting in a Neubauer haematocytometer. Three wells were prepared for the dye exclusion test in each experiment.

EFFECT OF HYDROGEN PEROXIDE CONCENTRATION ON THE PROLIFERATION OF TOTL-86 CELLS

In order to investigate the effects of hydrogen peroxide on TOTL-86 cells, different concentrations of hydrogen peroxide were added to the cells in culture. A 100 μl suspension of TOTL-86 cells in standard medium was plated into 96 well flat bottomed tissue culture plates (5000 cells/well) and allowed 16 hours to adhere. The medium was then replaced with 100 μl E-MEM with 0.5% FCS and then 1 μl of E-MEM, or E-MEM containing selected concentrations of hydrogen peroxide, was added to give a final concentration from 10^{-3} to 10^{-9} M.

To examine the effects of various growth factors on the proliferation of TOTL-86 cells, we chose EGF, bFGF, PDGF-AA, and insulin as the growth factors to study. We added 1 μl of E-MEM containing the selected growth factor (final concentration: EGF, bFGF, and PDGF-AA 10 ng/ml; insulin, 5 μg/ml) instead of hydrogen peroxide. After 48 hours, both the MTT assay and dye exclusion test were performed to evaluate the growth effects of each factor.

EFFECTS OF INITIAL CELL NUMBER ON THE SURVIVAL OF TOTL-86 CELLS

Because clinical reports have indicated that the number of residual lens epithelial cells was critical for the formation of secondary cataracts,[29–32] we evaluated the effect of the initial seeded cell number on the survival and growth of lens epithelial cells under constant

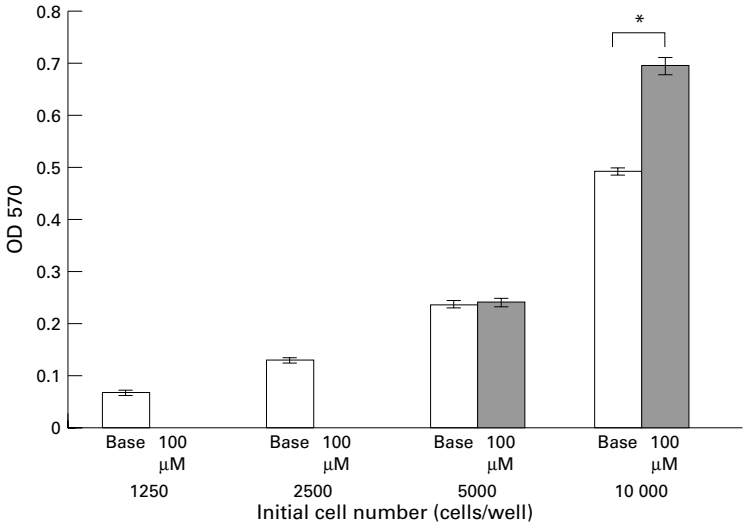

*Figure 3 The initial cell numbers on the influence of hydrogen peroxide. With low cell numbers, 1250 or 2500 cells/well, 100 μM hydrogen peroxide was cytotoxic. With 5000 cells/well, 100 μM hydrogen peroxide did not affect the proliferation. With 10 000 cells/well, 100 μM hydrogen peroxide was stimulating. Data represent the mean OD (SD) and * indicates statistical significance compared with base value (at 5000 cells/well, p = 0.5089: at 10 000 cells/well, p <0.0001).*

hydrogen peroxide concentration. We chose 100 μM of hydrogen peroxide for these experiments because this concentration was reported to be comparable with the 30 μM in normal human aqueous humour and with the higher levels found in patients with senile cataracts.[17–19] Standard medium (100 μl) containing 1250–10 000 cells/well was added to 96 well flat bottomed tissue culture plates and allowed 16 hours to adhere. The medium was then replaced by 100 μl E-MEM with 0.5% FCS containing 100 μM of hydrogen peroxide. After 48 hours, both the MTT assay and dye exclusion test were performed.

SYNERGISTIC EFFECT OF HYDROGEN PEROXIDE AND GROWTH FACTORS ON CELL PROLIFERATION

To investigate the synergistic effects of hydrogen peroxide and growth factors, we chose both 10 nM and 100 μM hydrogen peroxide to study. In 100 μl of standard medium, 5000 cells/well were plated in 96 well flat bottomed tissue culture plates and allowed 16 hours to adhere. Thereafter, the medium was replaced by 100 μl E-MEM with 0.5% FCS plus 1 μl of E-MEM containing both hydrogen peroxide (10 nM or 100 μM) and one of the selected growth factor (final concentration: EGF, bFGF, and PDGF-AA, 10 ng/ml; insulin, 5 μg/ml). After 48 hours, both the MTT assay and dye exclusion test were performed.

STATISTICAL ANALYSIS

The data are expressed as the mean (SD) OD obtained from three independent experiments (each experiment was performed in six replicate wells). Statistical significance was determined by the unpaired *t* test or linear regression analysis (STATVIEW J-4.5, Abacus Concepts, Inc, CA, USA), and differences were considered statistically significant when the p value was less than 0.01.

Results

Because of the good linear correlation (r=0.999, p<0.0001) between the seeded number of TOTL-86 cells and OD at 570 nm (Fig 1), we used the MTT assay for further analysis of the survival and proliferation of this cell line. In addition, OD at 570 nm and the corresponding counted cell numbers in each experiment were plotted as in Figure 1 (data not shown).

EGF, bFGF, PDGF-AA, and insulin stimulated the proliferation of TOTL-86 cell line significantly (Fig 2A) indicating that these cells had similar growth properties to other lens epithelial cells reported.[7 9 10]

EFFECT OF HYDROGEN PEROXIDE ON THE GROWTH OF TOTL-86 CELLS

As expected, a high level (1 mM) of hydrogen peroxide killed the TOTL-86 cells (Fig 2B) when measurements were made at 48 hours. In contrast, low levels (1 nM to 1 μM) of hydrogen peroxide stimulated the proliferation of TOTL-86 cells in a dose dependent manner (Fig 2B) and, interestingly, the cell count of the most effective level (1 μM) was comparable with that of the growth factors used in this study (see Fig 2A). When treated with 100 μM concentration of hydrogen peroxide, the number of TOTL-86 cells at 48 hours did not differ significantly from the initial number of cells. Thereafter, 100 μM was taken to be the sublethal concentration of hydrogen peroxide on TOTL-86 cells seeded at 5000 cells/well.

EFFECT OF INITIAL CELL NUMBERS ON THE SURVIVAL OF TOTL-86 CELLS

The initial number of seeded cells dramatically influenced the survival of TOTL-86 cells (Fig 3). With an initial low number of TOTL-86 cells (<5000 cells/well) the cells were not able to survive an exposure to the sublethal concentration (100 μM) of hydrogen peroxide. When seeded at 5000 cells/well, the cell numbers did not change when measured 48 hours after adding the 100 μM hydrogen peroxide (p = 0.5089). On the other hand, TOTL-86 cells cultured at higher initial cell number of 10 000 cells/well showed significant growth under the same sublethal concentration of hydrogen peroxide (p <0.0001).

SYNERGISTIC EFFECT OF HYDROGEN PEROXIDE AND GROWTH FACTORS ON TOTL-86 CELLS

We could not detect any synergistic effect on the proliferation of TOTL-86 cells when a low concentration of hydrogen peroxide (10 nM) was combined with the different growth factors (Fig 4A). However, when EGF and insulin were combined with the sublethal concentration of hydrogen peroxide (100 μM), there was an increase in the TOTL-86 cells at 48 hours (100 μM *v* 100 μM + EGF, p = 0.0003; 100 μM *v* 100 μM + insulin, p = 0.0002; Fig 4B). bFGF and PDGF-AA, on the other hand, did not alter the proliferation of the TOTL cells (100 μM *v* 100 μM + bFGF, p=0.0628; 100 μM *v* 100 μM + PDGF-AA, p = 0.0129).

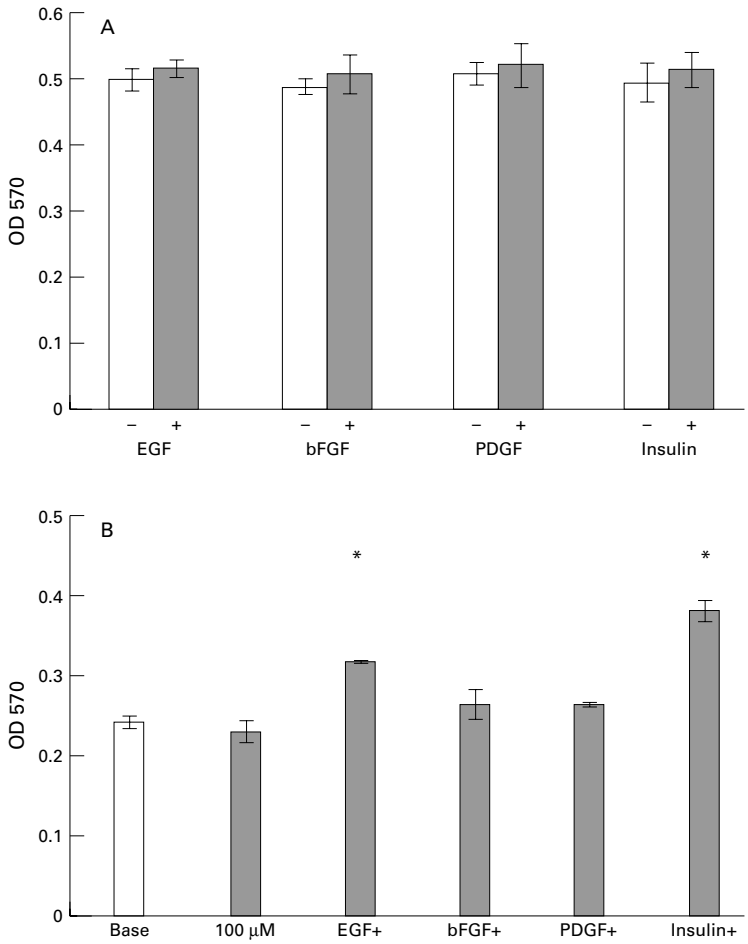

*Figure 4 (A) The synergistic effect of hydrogen peroxide and four growth factors. The concentration of hydrogen peroxide was 10 nM. No significant increase in the proliferation response of TOTL-86 cells is seen with any of the growth factors. "−" is the group treated with 10 nM hydrogen peroxide alone and "+" is the group treated with 10 nM hydrogen peroxide plus the growth factor. Unpaired t test was performed between the hydrogen peroxide alone v hydrogen peroxide + growth factor (10 nM v 10 nM + EGF, p = 0.2769: 10 nM v 10 nM + bFGF, p = 0.3825; 10 nM v 10 nM + PDGF, p = 0.4701; 10 nM v 10 nM + insulin, p = 0.4424). (B) The synergistic effect of hydrogen peroxide and four growth factors. The concentration of hydrogen peroxide was 100 μM. TOTL-86 cells treated with 100 μM hydrogen peroxide plus EGF or insulin showed significant cell proliferation compared with those treated with 100 μM hydrogen peroxide alone. In contrast, neither PDGF nor bFGF influence the growth response of TOTL-86 cells treated with 100 μM hydrogen peroxide. Columns labelled as EGF+, bFGF+, PDGF+ and insulin+ indicate that TOTL-86 cells treated with 100 μM hydrogen peroxide plus each growth factor. *Represents statistical significance compared with 100 μM hydrogen peroxide alone (100 μM v 100 μM plus EGF p = 0.0003; 100 μM v 100 μM plus insulin p = 0.0002; 100 μM v 100 μM plus bFGF, p = 0.0628; 100 μM v 100 μM plus PDGF, p = 0.0129).*

Discussion

In this study, we found that hydrogen peroxide can promote the mitotic activity of lens epithelial cells. However, the proliferation was concentration dependent with low doses leading to proliferation and high doses killing the epithelial cells.

Current studies have revealed that hydrogen peroxide can act as a signalling molecule under subtoxic concentrations.[12–16] Other studies have shown that hydrogen peroxide enhanced the tyrosine phosphorylation of the growth factor receptor.[33–35] This may explain why the maximum cell numbers attained under the optimal concentration of hydrogen peroxide (1 μM) was comparable with that of the growth factors. Other studies have shown that a variety of cells enter into a long term growth arrest under sublethal concentrations of hydrogen peroxide.[36 37] This is comparable with the findings with the sublethal concentration of hydrogen peroxide observed in this study.

We also found that the effects of hydrogen peroxide depended on the initial seeded number of lens epithelial cells. This may be explained by the detoxification of hydrogen peroxide by lens epithelial cells because lens epithelial cells have the glutathione system to degrade hydrogen peroxide.[38–41] In addition, several investigators have reported that lens epithelial cells secreted some autocrine factors which promote the survival and the proliferation of lens epithelial cells,[42 43] and Davidson *et al* have demonstrated that the antioxidant transferrin is one of them.[44] Increasing the number of cells seeded would increase the rate of detoxification. Hence higher density shifted the dose response curve to the left.

One other interesting finding in this study was the observation that both insulin and EGF, besides controlling cell proliferation, might have another effect on lens epithelial cells. We observed that both insulin and EGF, but not bFGF and PDGF-AA, led to a proliferation of TOTL-86 cells when exposed to the sublethal concentration of hydrogen peroxide. Recently, Yang and de Bono demonstrated that VEGF and bFGF increased the resistance of human umbilical vein endothelial cells to oxidative stress.[45] They reported the generation of an active defence mechanism involving the formation of reducing equivalents of NADPH by glucose metabolism via the pentose phosphate pathway, and glutathione and glutathione peroxidase were upregulated by VEGF and bFGF in endothelial cells. It is well known that lens epithelial cells also have a similar active defence mechanism against oxidative stress.[38–41] We can thus suggest that both EGF and insulin, especially insulin, may have the same effect on lens epithelial cells.

While it has long been speculated that oxidative stress has an important role in cataractogenesis, the present study provides an additional perspective that secondary cataract may also be regulated by hydrogen peroxide through a unique cooperation with some growth factors. To understand this cooperative mechanism may open up a new avenue to prevent secondary cataract formation.

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